Availability of brachymorphic mice as undersulfated animals.

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Brachymorphic mice have a chondrodystrophia that is characterized by inheritable dwarfism with disproportionally shortened limbs, tail and trunk. This malformation is induced by a bm gene that is a recessive gene on the autosomal chromosome, and generates a point mutation in bifunctional sulfurylase kinase polypeptide functioning as ATP-sulfurylase and adenosine-phosphosulfate kinase. In other words, this disorder in the mice arises from the undersulfation of cartilage tissues with the decreasing synthesis of sulfate donor by the abnormal sulfurylase kinase. Because the sulfurylase kinase is universally distributed in the tissues which require sulfate groups in the sulfation pathway, it is possible that the bm gene induces the undersulfation in another tissues as well as in the cartilage tissues. Consequently, we transferred the bm gene from the brachymorphic mice of the C57BL strain to the normal mice of the BALB/c strain which we routinely use as experimental animals. In both the normal and brachymorphic mice of the BALB/c strain, paraformaldehyde-fixed and paraffin-embedded sections were routinely prepared from intestines, kidney, lung, tibia and vertebra. Sections were stained with the sensitized high iron diamine procedure for detecting acidic groups and alcian blue staining with critical electrolyte concentration for analyzing the concentration of acidic groups in tissues. From the light-microscopic observations, staining intensities of acidic groups in basement membrane of various tissues, extracellular matrices of cartilage tissues in tibia and vertebra, and goblet cells in the small intestine and the colon decreased in the brachymorphic mice, as compared with the normal mice. Conclusively, we confirmed that brachymorphic mice of BALB/c strain are available as undersulfated experimental animals. Additionally, brachymorphic mice of BALB/c strain are possible to be a model animal such as depression of anionic barrier in basement membrane, except for chondrodysplasia.

Key words (five):
brachymorphic mouse, undersulfation, experimental animals, glycosaminoglycans, histochemistry

Introduction

Sulfation is one of the critical functions in a variety of biomolecules such as proteins, carbohydrates and lipids. In vertebrates, it has been known that the sulfation requires the enzymatically synthesized activating sulfate donor, phosphoadenosine-phosphosulfate (PAPS) [1]. The sulfate activation pathway consists of two activities; ATP-sulfurylase, which catalyzes the synthesis of adenosine-phosphosulfate (APS) from ATP and SO_4^{-2}, and APS kinase, which phosphorylates APS in the presence of another molecule of ATP to form PAPS[2].

Lane and Dickie[3] first reported homozygous brachymorphic mice that have an inherited disorder affecting cartilage structure and function. Brachymorphic mutation in mice is characterized by dwarfism that involves malformations such as disproportionally shortened limb bones, a short thick tail and a dome-shaped spinal column[3]. This mutation is caused by a recessive single mutant gene (bm gene) on the autosomal chromosome (mouse chromosome 19)[1,3].Afterward, in the bm homozygote mice, it has been found that an enzymatic synthesis of PAPS is disordered[4-8]. And recently, it has been discovered that the bm gene induces a point mutation of bifunctional sulfurylase kinase having both ATP-sulfurylase and adenosine-phosphosulfate kinase activities[1]. In conclusion, the decreasing synthesis of PAPS causes undersulfation of chondroitin sulfate in the cartilage matrix and affects chondorogenesis disorder (chondrodystrophia), resulting in the brachymorphic mutation in these mice [9-13].

So far numerous attempts have been made by scholars to clarify the character, development and etiology of the brachymorphism in the mouse, as described above. However, most of biochemical and morphological studies have focused mainly on the limb cartilage in the brachymorphic mice. Since the sulfurylase kinase and PAPS were widely distributed in various tissues that have a sulfation pathway, it is possible to induce the undersulfation in other tissues as well as in cartilage tissues. However, no reports focusing on other tissues in the original brachymorphic

mice of the C57BL strain have ever presented.

Original purpose is to make an experimental animal having undersulfated heparansulfate in vascular basement membrane, to investigate that origin of blood vessel is either host animal (BALB/c mice) or metastatic cancer in the vascularization in cancer metaplasia. Consequently, we transferred the bm gene from the brachymorphic mice of the C57BL strain to the normal mice of the BALB/c strain which we routinely use as experimental animals. The purpose of this paper is to histochemically search the undersulfation in various tissues of the brachymorphic mice of the BALB/c strain and to discuss the availability of the mice as undersulfated experimental animals.

Materials and methods

Establishment of brachymorphic mice in the BALB/c strain: The bm mice of BALB/c strain were created by the transferring the bm gene from original brachymorphic mice (C57BL/6-bm/bm) to the BALB/c strain with outbreeding. In the first step, a male of C57BL/6-bm/bm was caged with a female of normal BALB/c strain (BALB/c-+/+). In the second step, hybrids obtained from C57BL/6-bm/bm and BALB/c-+/+ were inbred in the litter. In the third step, a young inbred mail that had a brachymorphism was outbred with a female BALB/c-+/+ again and hybrids were obtained. We performed the process described above (steps 2-3) eight times. In the fourth step, a female BALB/c-bm/bm was caged with a male BALB/c-+/+ and hybrids were obtained. In the fifth step, the hybrids were inbred in the litter. Finally, the obtained mice that have the brachymorphic mutations were considered to be first generation of BALB/c-bm/bm and inbred. In the present study, we used the 22-24th generation of BALB/c-bm/bm mice for the materials.

Tissue preparation: 10 males of normal (BALB/c-+/+) and brachymorphic (BALB/c-bm/bm) mice were sacrificed at the age of 6 weeks after birth. All experimental animals were treated in accordance with the guidelines for the care and use of laboratory animals in Nagoya Bunri University. After undergoing the pentobarbital anesthesia, the mice were perfused via the left cardiac ventricle with physiological saline solution followed by perfusion fixation of 4.0% paraformaldehyde in 0.05M phosphate buffer (pH 7.4) solution containing 7.5 % sucrose. After perfusion fixation, small intestine, colon, kidney, lung, tibia and thorax vertebra were dissected out, immersed in the same fixative solution used in the perfusion at 4 °C for 3-7 days, and then the tissues were rinsed with 0.05 M phosphate buffer (pH 7.4) containing 0.9% sodium chloride. Some calcified tissues such as tibia and vertebra were decalcified by 2.5 % ethylenediamine tetraaccetic acid solution, which was neutralized with sodium hydroxide and added 7.0% sucrose, for 10-30 days at 4 °C and rinsed thoroughly with phosphate buffer containing sucrose. Then, all of tissues were dehydrated with an ethanol series of ascending concentration (70%-100%), cleared with xylene and embedded in paraffin wax (melting point: 58-60 °C). The sections were cut at a thickness of approximately 4 μm, mounted on silane-coated (3-aminopropyl triethoxysilane: Tokyo Kasei Kogyo Co., Tokyo, Japan) slide glass and dried in a hot oven at 37 °C for 5-6 hours.

Histochemical staining: To detect acidic glycoconjugates in the tissues, the sensitized high iron diamine (S-HID) method was employed [14]. The S-HID method has better sensitivity and visualization for sulfate groups than other detecting methods, and it can detect very small amounts of acidic glycoconjugates in tissues. A detailed procedure of the S-HID method has been described in Hirabayashi previously[14]. Deparaffinized and hydrated sections were incubated in a high iron diamine solution at 30°C for 60 minutes, immersed in 0.5mM potassium trichloro(ethylene) platinum solution at room temperature for 60 minutes, reduced by 0.2% sodium borohydride solution at room temperature for 30 seconds, subjected to a silver enhancement procedure at 20°C for 5-8 minutes, and then incubated in an appropriate photographic fixer at room temperature for 2 minutes one after the other. Finally, sections were counter-stained with hematoxylin. Between each step, sections were washed with tap water and distilled water thoroughly. In addition, to identify the molecular species of glycosaminoglycans in tissues, some sections were digested with glycosaminoglycan-degrading enzymes such as chondroitinase (Chase) B[15-18] and testicular hyaluronidase (T-Hylase)[17-20,]. Detailed conditions of enzyme digestions were described in Table 1.

Table 1: Condition of enzyme digestions.

Enzymes	Origin	Substrate specificity	Incubating conditions
chondroitinase B (Chase B)	Flavobacterium heparinum (Seikagaku Kogyo Co., Tokyo, Japan)	DS	0.1unit/ml of Chase B in 0.1M Tris-HCl buffer (pH 8.0) at 30 °C for 18-24 hours.
testicular hyaluronidase (T-Hylase)	bovine testis (Type VIII) (Sigma Chemical Co., St. Louis, MO, USA)	CS (A and C), CH, HA	1.0 mg/ml of T-Hylase in 0.1M phosphate buffer (pH 5.5) at 37 °C for 18-24 hours.

DS: dermatan sulfate, CS: chondroitin sulfate, CH: chondroitin, HA: hyaluronic acid.
As controls for the enzyme digestion experiments, two procedures were used: a) some sections were kept intact without any incubation procedures and b) others were incubated in each buffer solution without enzymes under identical temperature and for the same duration of time.

To demonstrate the ion strength of acidic groups in tissues, alcian blue staining with critical electrolyte concentration (AB-CEC)[21] was employed. A series of deparaffinized and hydrated sections were incubated in 0.1% AB in 0.05M sodium acetate buffer at pH 5.7 containing different concentration of magnesium chloride (0, 0.2, 0.4, 0.6 and 0.8 M) at room temperature overnight. After AB-CEC staining, without washing with tap water and counter staining, all sections were dehydrated with ethanol.

After the staining described above, all sections were dehydrated with ethanol series of ascending concentrations, cleared in xylene and covered with glass slips using an HSR solution (Harleco synthetic resin: Kokusi Shiyaku Co., Kobe, Japan).

Results

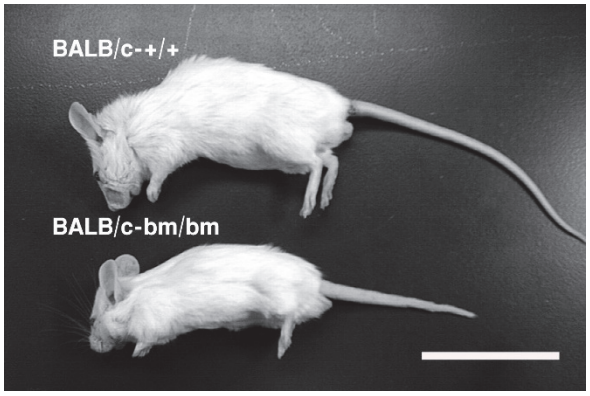

Fig. 1; Photograph of the normal mouse (BALB/c-+/+) (upper) and the brachymorphic mouse (BALB/c-bm/bm) (lower). In the brachymorphic mouse, because the growth of cartilage in osteochondrogenic differentiation is inhibited in longitudinal direction, long bones of arm and leg become short as well as vertebral bone. As results, the morphological character of brachymorphic mouse is the dwarfism such as shortened limb, trunk and head, and thick and shortened tail. Bar=5 cm

Appearances of the normal (BALB/c-+/+) and brachymorphic (BALB/c-bm/bm) mice were shown in Figure 1. The malformations such as disproportionally shortened limbs and trunk, and a short thick tail characterized the dwarfism of brachymorphic mice (Fig. 1).

Observation of tissue specimens stained with the S-HID method: With S-HID staining, many tissue components containing sulfate groups in the small intestine and the colon of normal mice, such as goblet cells, basement membranes of epithelium and connective tissues of lamina propria, submucosa and inter muscular matrices, indicated medium to strong positive reactions (dark brown to black color in shade) (Figs. 2A and 2B). The staining intensities of goblet cells and connective tissues of lamina propria, submucosa and inter muscular matrices in the brachymorphic mice were weaker than those in the normal mice (Figs. 2E and 2F). In these findings, especially, it should be noted that some goblet cell mucins in the brachymorphic mice indicated weak positive reactions (brown in colors) with S-HID. After the digestion with Chase B and T-Hylase for removing the isomeric chondroitin sulfates, basement membranes of renal glomeruli and urinary tubules exhibited marked positive reactions in the normal mice (Fig. 2C). On the other hand, basement membranes of same tissue components in the brachymorphic mice indicated weak to moderate positive reactions to S-HID (Fig. 2G). In the lung, Chase B and T-Hylase digestion-resisting and S-HID positive tissue structures were basement membranes of blood vessels and alveoli in the normal mice (Fig. 2D). These basement membranes in normal mice showed moderate to strong positive reaction of S-HID. However, in the brachymorphic mice, the staining intensities of the basement membrane in same tissues were weaker than those in the normal mice (Fig. 2H). In the proximal epiphyseal plate of tibial bone, inter cellular matrices of cartilage indicated markedly strong positive reactions to S-HID in both the normal and the brachymorphic mice (Figs. 2I and 2M). However, chondrocytes were smaller in the brachymorphic mice than in the normal mice, especially in the hypertrophic cell layer. After the digestion with

T-Hylase, the staining reactions of inter cellular matrices were decreased and showed weak positive reaction in the normal mice (Fig. 2J). On the other hand, the digestion with T-Hylase abolished the staining intensities of intercellular matrices in the brachymorphic mice (Fig. 2N). Cartilage matrices of endplate in vertebral bones and inner and outer annuluses of intervertebral disks indicated vividly strong positive reaction of S-HID in both the normal and the brachymorphic mice (Figs. 2K and 2O). After the double digestion with Chase B and T-Hylase, the staining intensities of S-HID in these cartilage matrices markedly decreased in both the normal and brachymorphic mice (Figs. 2L and 2P). The S-HID positive reactions of the double digestion-resisting tissue components in the normal mice such as endplate and inner annulus were stronger than those in the brachymorphic mice (Figs. 2L and 2P).

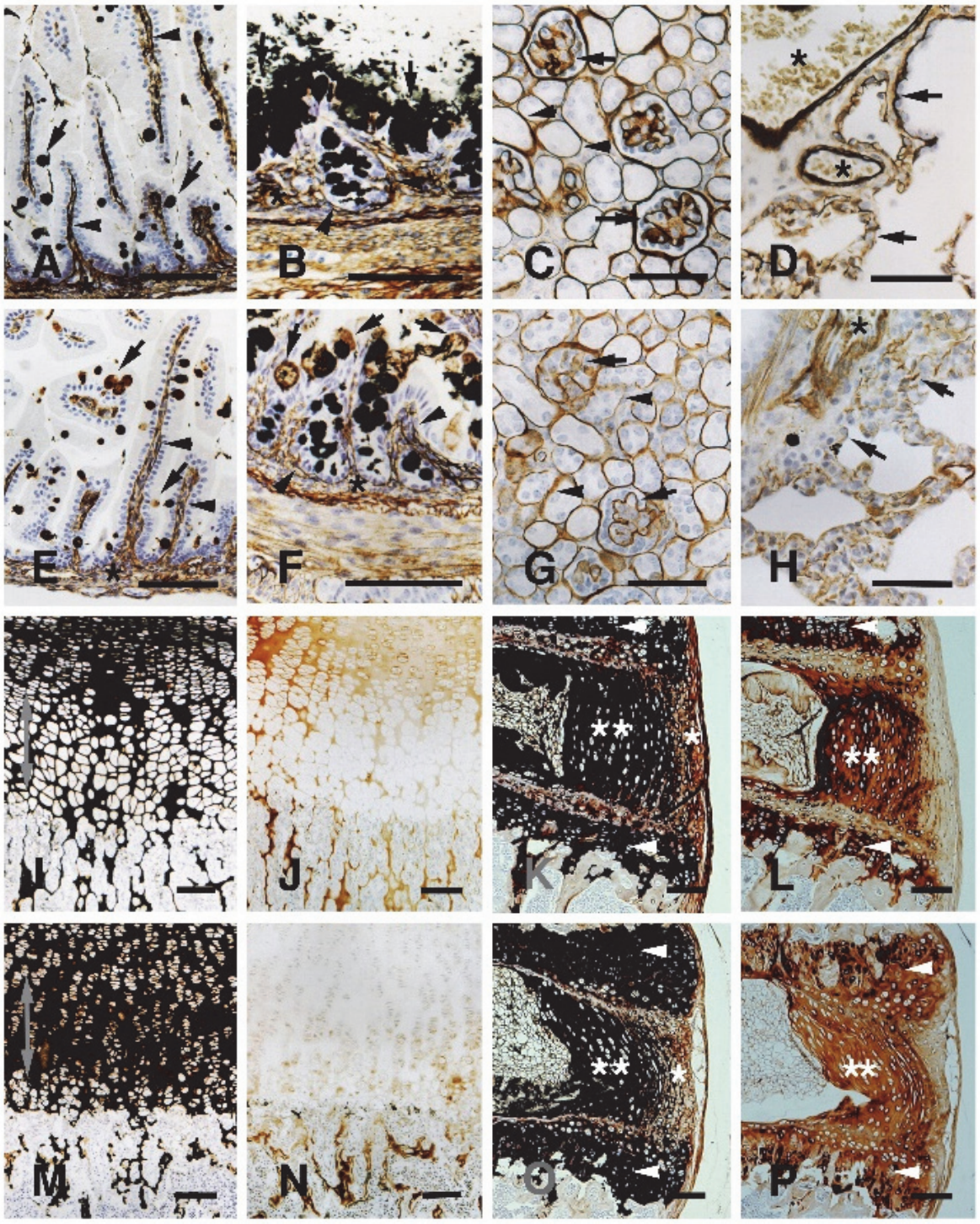

Fig. 2; Photomicrographs of different tissues in normal (A-D and I-L) and brachymorphic (E-H and M-P) mice. A and E: small intestine, S-HID staining. Bar = 100 μm. B and F: colon, S-HID staining. Bar = 100 μm. In Figures A, B, E and F, arrows, arrowheads and asterisks show goblet cell mucins, basement membranes of epithelium and lamina propria connective tissues, respectively. C and G: kidney, S-HID staining treated with double enzyme digestions of Chase B and T-Hylase. Arrows and arrowheads indicate basement membranes of glomerulus and urinary tubules, respectively. Bar = 100 μm. D and H: lung, S-HID staining treated with double enzyme digestions of Chase B and T-Hylase. Asterisks and arrows show blood vessels and basement membranes of alveoli, respectively. Bar = 100 μm. I, J, M and N: proximal epiphyseal plate of tibial bone, S-HID staining with (J and N) or without (I and M) an enzyme digestion of T-Hylase. In I and M, areas that were indicated by arrows show hypertrophic cell layer. Bar = 100μm. K, L, O and P: vertebral bone and intervertebral disc, S-HID staining with (L and P) or without (K and O) double digestion of Chase B and T-Hylase. Arrowheads indicate endplates of vertebral bone. In the intervertebral disc, outer (*) and inner (**) annulus were shown. indicate Bar = 100 μm.

Observation of tissue specimens stained with the Alcian blue with critical electrolyte concentration: In the normal mice, mucins of goblet cells in the colon were strongly stained with AB without magnesium chloride (Fig. 3A). By the staining with AB containing 0.2M magnesium chloride, staining intensities of goblet cell mucins were slightly decreased (Fig. 3B). Under the addition of 0.4M magnesium chloride, the staining intensities of AB in the mucins decreased markedly (Fig. 3C). In the staining of AB containing 0.6M magnesium chloride, the staining intensities of the goblet cell mucins were abolished absolutely (Fig. 3D). In the brachymorphic mice, when the colon tissues were stained with magnesium chloride-free AB solution, mucins of goblet cells showed strong positive reactions similar to those in normal mice (Fig. 3E). Because of the existence of 0.2M magnesium chloride, the staining intensities of goblet cell mucins were decreased markedly (Fig. 3F). Under the condition of AB containing 0.4-0.6M magnesium chloride, the staining intensity of goblet cell mucins were completely abolished (Fig. 3G and 3H).

In the normal mice, the intercellular matrices of proximal epiphyseal plate in tibial bone indicated moderate to strong positive reaction by means of AB staining without magnesium chloride (Fig. 3I). By the staining with AB containing 0.4-0.6M magnesium chloride, staining intensities of intercellular matrices were decreased moderately (Figs. 3J and 3K). In the staining of AB containing 0.8M magnesium chloride, the staining intensities of the matrices were mostly abolished (Fig. 3L). In the brachymorphic mice, the intercellular matrices in proximal epiphyseal plate of tibial bone showed variety of (moderate to strong) positive reaction with the staining of AB without magnesium chloride (Fig. 3M). When the proximal epiphyseal plate was stained with AB containing 0.4M magnesium chloride, the staining intensities of the intercellular matrices decreased clearly (Fig. 3N). Under the presence of 0.6M magnesium chloride, AB-staining reactions of intercellular matrices mostly disappeared (Fig. 3O). Then, by the AB staining with 0.8M magnesium chloride, the staining reactions of cartilage matrices in the proximal epiphyseal plate of the brachymorphic mouse were abolished absolutely (Fig. 3P).

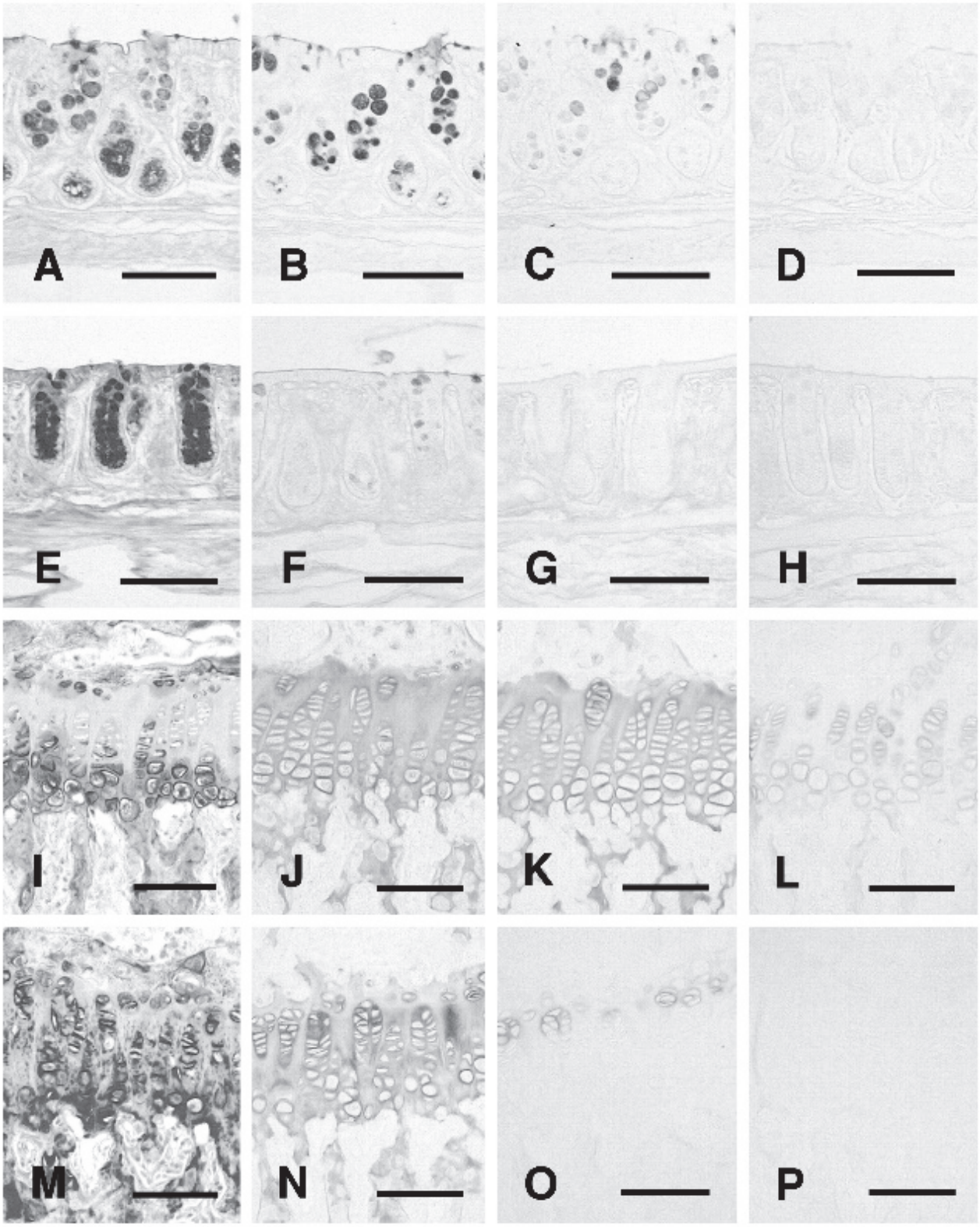

Fig. 3: Photomicrographs of different tissues in normal (A-D and I-L) and brachymorphic (E-H and M-P) mice. A-H: colon, AB staining containing 0 (A and E), 0.2 (B and F), 0.4 (C and G) and 0.6 (D and H) M magnesium chloride. Bar = 100 µm. I-P: proximal endplate of tibial bone, AB staining containing 0 (I and M), 0.4 (J and N), 0.6 (K and O) and 0.8 (L and P) M magnesium chloride. Bar =100 µm.

Discussion

The staining system of S-HID was constructed with the high iron diamine procedure and silver enhancement[14]. Since the detecting mechanisms of acidic groups are based on the high iron diamine procedure developed by Spicer[22], the changing colors from brown to black of S-HID depends on the existing amounts of sulfated groups in tissues. In the present study, in order to identify the molecular species of glycosaminoglycans, glycosaminoglycan-degrading enzymes were employed. In order to classify isomeric chondroitin sulfates, chondroitinase B (Chase B) and testicular hyaluronidase (T-Hylase) were chosen (detail was shown in Table 1). Through S-HID staining specificity[14] and substance specificity of the enzyme digestions with Chase B[15-18] and T-Hylase[17-20], the molecular species of glycosaminoglycans involved in the tissues could be identified clearly.

Scott and coworkers described staining mechanisms and basic theory of AB-CEC[21,23]. The critical electrolyte concentration in glycosaminoglycans (GAGs) is decided by the ratio of sulfate- and carboxyl- groups involved in the GAG molecule, and is the inherent value in each GAG molecule. Since the staining mechanism of AB-CEC is a simple ion exchange, the equilibrium depends on the relative affinities of the dye (AB) and inorganic cation (magnesium ion) for the charged groups of the anionic site in the tissues. In other words, AB-reacting sites of the GAG molecules in the tissues were blocked by the competition between the relative affinities of AB and magnesium iron in the solution for acidic groups in tissues. Therefore, the molecules that need a high concentration of magnesium chloride to abolish AB staining can contain large amounts of acidic groups. According to Scott and coworkers[21,23], the values of critical electrolyte concentration of magnesium chloride for AB staining reactions are 0.2M for carboxyl groups, 0.6-0.8M for sulfate groups in chondroitin sulfates and over 1.0M for sulfate groups in keratan sulfates. In the present study, the values of critical electrolyte concentration of magnesium chloride in all employed tissues of the brachymorphic mice were lower than those of the normal mice. The results indicate that effects of disordered sulfate kinase are not confined to the cartilage tissues and widely distributed in variety of tissues in the brachymorphic mice of the BALB/c strain.

It has been well known that the intercellular matrices of connective tissues contain a variety of glycosaminoglycans (GAGs) such as hyaluronic acid, dermatan sulfate and chondroitin sulfates[14,24], and that the goblet cell mucins involve acidic glycoprotein such as sulfosialomucin[14,25]. According to the present results from S-HID and AB-CEC procedures, it is found that the glycoprotein in the goblet cell mucins and glycosaminoglycans in lamina propria, submucosa and intermusclar connective tissues of small intestine and colon were undersulfated in the brachymorphic mice. Interestingly, the special note was that the acidic glycoconjugate of the goblet cell mucins was undersulfated in the brachymorphic mice of BALB/c strain. So far as we know, these findings have not been reported in brachymorphic mice yet. Kurima et al. described that undersulfation in brachymorphic mice of C57BL were observed in selected tissues such as cartilage and liver[1]. According to Schwartz and Domowicz[26], the mammals usually have two types of the sulfate kinase (APS-sulfurylase/PAPS kinase) isozyme (SK1 and SK2). And brachymorphic mice of C57BL has normal SK1 and abnormal SK2 that has one point mutation in the amino acid sequence[1], so that brachymorphic mice of BALB/c likely has normal SK1 and abnormal SK2, correspondingly. We speculate that the expression pattern of sulfate kinase isozymes (SK1 and SK2) is different between the brachymorphic mice of C57BL and BALB/c strains. On the effects of undersulfation in the gastrointestinal tract, one report states the relationship between mucosal sulfated carbohydrates and adult worms, Strongyloides venezuelensis [27]. In the report, when the adult worms were implanted in the small and large intestines of brachymorphic and normal mice, significantly greater numbers of adult worms could settle in the large intestine of brachymorphic mice, as compared to normal mice. In large intestine, goblet cell mucin contains large amount of acidic glycoconjugates, sialo-, carboxyl- and sulfated mucins. The mucin in large intestine protects hosts from infection with virus, bacteria and parasite. This report suggests that undersulfation of goblet cell mucin in large intestine compromised in the infection of worms. Consequently, the undersulfation in the goblet cell mucins affects the biophylactic system in the gastrointestinal tracts[27].

In the observation of kidney and lung in the present study, most of the remaining S-HID positive reaction in basement membrane might be heparan sulfates after isomeric chondroitin sulfates were eliminated by double digestion with Chase B and T-Hylase. Moreover, the basement membrane of the small intestine and the colon in the brachymorphic mice indicated weak positive reactions of S-HID without any enzyme digestions. Heparan sulfate is a principal component of basement membrane, and plays the important role of an anionic barrier and growth factor trapping[28-30]. Since S-HID staining of kidney and lung in brachymorphic mice indicated weak positive reactions as compared with those of

normal mice, it is speculated that GAGs such as heparan sulfate involved in basement membrane may be undersulfated. Moreover, according to the results from the small intestine and the colon treated with S-HID procedure, most of acidic glycoconjugates in the basement membrane are thought to be undersulfated. The undersulfation of basement membranes is crucial for the metabolic and functional properties of the tissues, and effective for not only the transport of fluid and ions, but also for cell invasion and migration[31,32]. The undersulfation of basement membrane was observed in the variety of tissues in the brachymorphic mice of BALB/c strain. Therefore, the undersulfation of basement membrane could affect metastasis of cancer cell, parasitism of worm in gastrointestinal tract, selective filtration of glomerulus in kidney and all.

Generally, it has been known that cartilage matrix contains aggrecan, major cartilage proteoglycan which consists of core protein and glycosaminoglycan chain such as isomeric chondroitin sulfates and keratan sulfate[24,33]. In the present study, the staining intensity of S-HID in the cartilage tissues of epiphyseal plate in tibial bone, endplates in vertebral bone and outer and inner annulus in intervertebral disc of the brachymorphic mice is almost same as those of the normal mice. However, AB-CEC procedure revealed the undersulfation of intercellular matrices in cartilage tissues of the brachymorphic mice. In the results from AB-CEC procedure, abolishment of AB staining in cartilage matrices of brachymorphic mice by the adjunction of 0.6M magnesium chloride is highly important. This finding indicates that cartilage matrices of the brachymorphic mice contain undersulfated chondroitin sulfates and that the matrices does not involve keratin sulfates or contain exceedingly vary few amount of undersulfated keratin sulfates. The reason for this speculation is that AB-staining reactions of normal chondroitin sulfate and keratan sulfate resist the adjunction of 0.6M and 0.8M magnesium chloride respectively[21,23]. After enzyme digestion to reveal isomeric chondroitin sulfates, enzyme–resisting and S-HID positive reactions of cartilage matrices in the normal mice were stronger than those in the brachymorphic mice. In view of the GAG components in cartilage tissues[24,33], the remaining substances in the cartilage that resist the isomeric chondroitin sulfate-degrading enzyme are thought to be mainly keratan sulfate. According to the results from AB-CEC and S-HID procedure, it is speculated that cartilage matrices of brachymorphic mice could be involved in undersulfated chondroitin sulfate and relatively decreasing amounts of keratan sulfate or undersulfated one. In other words, the GAG components of cartilage matrices in the brachymorphic mice are different from the normal mice. Therefore, it is thought that the abnormal components of GAGs in cartilage matrices disrupt the functional integrity of cartilage tissues. In other works, the material disorder affects the biological and biomechanical function of hyaline and fibrous cartilages, such as bone development, resistance to the mechanical loading, response in the mechanotransduction and collagen assembly[34-37]. And it seems that these abnormalities induce the malformation, brachymorphism in the BALB/c mice.

Conclusively, it is demonstrated that the brachymorphic mice of BALB/c strain have the undersulfation in a variety of tissues. We think that the comparison between normal sulfation and undersulfation can be useful for discussing the function of sulfation in animals. According to the discussion in the paper, we think that the brachymorphic mice of the BALB/c strain have value in experiments as undersulfated animals.

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